High expression of the mismatch repair protein MSH6 is associated with poor patient survival in melanoma

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Abstract: OBJECTIVES: The outcome of patients with primary melanoma (PM) cannot be completely explained based on currently adopted clinical-histopathologic criteria. In this study, we evaluated the potential prognostic value of mismatch repair protein expression in PMs. METHODS: We examined the immunohistochemical staining of mismatch repair proteins in 18 benign nevi and 101 stage I to III PMs and investigated their association with tumor clinicopathologic variables and melanoma mortality. RESULTS: Expression of MSH2, MLH1, and PMS2 was high in benign nevi and reduced in a subset of PMs. Conversely, MSH6 expression was absent or extremely low in benign nevi and increased in a subset of PMs. In the multivariate analysis, including sex, age, Breslow thickness, and ulceration, high MSH6 expression in PMs (ie, immunostaining in >20% of tumor cells) was significantly associated with an increased risk of melanoma mortality (relative risk, 3.76; 95% confidence interval, 1.12-12.70). CONCLUSIONS: MSH6 protein expression can be a valuable marker to improve prognosis assessment in PMs.

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CME/SAM

High Expression of the Mismatch Repair Protein MSH6 Is Associated With Poor Patient Survival in Melanoma

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Key Words: Mismatch repair; MSH6; Melanoma; Survival

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ABSTRACT

Objectives: *The outcome of patients with primary melanoma (PM) cannot be completely explained based on currently adopted clinical-histopathologic criteria. In this study, we evaluated the potential prognostic value of mismatch repair protein expression in PMs.*

Methods: *We examined the immunohistochemical staining of mismatch repair proteins in 18 benign nevi and 101 stage I to III PMs and investigated their association with tumor clinicopathologic variables and melanoma mortality.*

Results: *Expression of MSH2, MLH1, and PMS2 was high in benign nevi and reduced in a subset of PMs. Conversely, MSH6 expression was absent or extremely low in benign nevi and increased in a subset of PMs. In the multivariate analysis, including sex, age, Breslow thickness, and ulceration, high MSH6 expression in PMs (ie, immunostaining in >20% of tumor cells) was significantly associated with an increased risk of melanoma mortality (relative risk, 3.76; 95% confidence interval, 1.12-12.70).*

Conclusions: *MSH6 protein expression can be a valuable marker to improve prognosis assessment in PMs.*

Upon completion of this activity you will be able to:
- evaluate mismatch repair (MMR) protein expression in benign nevi and melanoma specimens by immunohistochemistry.
- define the dominant prognostic factors for patients with primary melanoma.
- define which MMR protein is significantly associated with an increased risk of melanoma mortality.

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Questions appear on p 134. Exam is located at www.ascp.org/ajcpcme.

Cutaneous melanoma is an extremely aggressive malignant disease, endowed with high metastatic potential and affecting predominantly young people. Moreover, with the exception of lung cancer in women, its worldwide incidence is increasing faster than that of any other neoplastic disease.[1,2]

Presently, the prognosis of a patient with cutaneous melanoma is essentially derived from clinical and histopathologic parameters. The dominant prognostic factors for patients with localized or regional metastatic melanoma are represented by primary tumor thickness, ulceration, number of mitoses/mm^2 (for tumors ≤1.0 mm), and the number of tumor-positive nodes and their tumor burden.[3] All these parameters are incorporated in the latest version of the American Joint Committee

on Cancer (AJCC) staging system for melanoma.[3] According to survival data from the AJCC melanoma database, the probability of 5-year survival for patients with localized stage I melanoma is higher than 90%, whereas that for patients with stage II disease ranges between 45% and 77%.[3] Once metastasis to regional lymph nodes occurs (stage III), the 5-year survival ranges from 24% to 69%.[3] However, due to the high biological heterogeneity of melanoma, an individual patient's prognosis cannot be accurately predicted by the currently adopted clinical-histopathologic criteria, and therefore there is a pressing need to identify new molecular markers that may aid risk stratification of patients with stage I to III melanoma and hence improve selection of those patients to be enrolled for adjuvant therapy.[4,5]

During the past decade, high-throughput technologies evaluating expression levels of protein-coding genes have been used extensively in cancer research to uncover critical molecular events underlying disease development and progression, as well as to identify new potential prognostic markers and therapeutic targets. In this regard, several studies have been performed on melanoma cell lines or specimens to identify gene expression signatures associated with tumor cell metastatic potential (ie, invasiveness signatures), patient disease-free survival, and/or overall survival (ie, prognostic signatures).[6-7] These investigations, as a consequence of their heterogeneity in terms of sample (ie, cell lines, primary melanoma, or metastases) and study population, as well as array platforms and biostatistical methods used, have generated a number of different prognostic or invasiveness signatures containing a limited number of overlapping genes.[6,7] Nevertheless, when biological processes are considered, most of the identified signatures show that genes differentially expressed between invasive and noninvasive melanomas or associated with distinct clinical outcomes are mainly involved in the regulation of apoptosis, cell cycle progression, DNA duplication and repair, epithelial-mesenchymal transition, and immune response.[6,7] In particular, elevated levels of DNA repair gene transcripts, including those of several genes participating in mismatch repair (MMR), have been associated with unfavorable histopathologic features and/or poor clinical outcome in four of the seven studies performed on primary melanomas to identify prognostic gene signatures.[8-14]

Although gene expression profiling has provided useful insight into the molecular mechanisms involved in melanoma development and progression and most likely will improve disease management in the era of personalized medicine, it is not yet practicable for the routine prognostic assessment of patients with melanoma. On the other hand, immunohistochemistry can be equally informative in characterizing the expression pattern of specific proteins in formalin-fixed, paraffin-embedded tumor specimens while preserving tissue and cellular architecture. Confirmation of gene expression profiling data at the protein level using immunohistochemistry and assessment of protein expression association with patient outcome could be therefore a valuable strategy to identify new potential prognostic markers. On these bases, in the present investigation, we evaluated the expression of the four key proteins involved in MMR—namely, MSH2, MSH6, MLH1, and PMS2[15,16]—in both benign nevi and a large series of stage I to III primary melanomas. Thereafter, we investigated the association between protein expression in the malignant lesions and clinicopathologic variables and 10-year melanoma mortality. Actually, elevated levels of *MSH2* and/or *MSH6* messenger RNA (mRNA) have been detected in primary melanomas with poor prognosis by different studies.[8,10,12,13]

Materials and Methods

Patients and Specimens

The study, a hospital-based retrospective cohort study, was conducted on 101 patients who underwent surgical resection of a sporadic primary cutaneous melanoma at the Istituto Dermopatico dell'Immacolata-IRCCS (IDI) from January 1995 through December 2001. The study also included 18 patients who underwent surgical resection of a compound melanocytic nevus (CMN). These patients were selected to be comparable to the group of patients with melanoma in terms of the percentage of males and females as well as in terms of age range. Characteristics of all patients and primary melanomas are shown in **Table 1**. The study was conducted according to the Declaration of Helsinki principles and approved by the Ethical Committee of the IDI (No. 148/CE/2011; December 16, 2011). Written informed consent was obtained from all patients.

Archival H&E-stained sections of all lesions were reexamined by one pathologist (F.P.), and the following histologic features were recorded for the melanoma specimens: (1) histologic type, (2) Breslow thickness, (3) mitotic rate (ie, number of mitoses/mm^2), and (4) presence or absence of regression or ulceration. The presence of an increased number of blood vessels or scattered lymphocytes and melanin-laden macrophages together with lamella fibrosis in the dermis close to the tumor was recorded as a sign of regression.[17] Ulceration was considered present if there was full-thickness loss of the epidermis.

Seven clinical parameters were included in the database used to conduct the study: age, sex, location of primary melanoma, date of diagnosis, clinical stage at the date of diagnosis, cause, and date of death. Anatomical location of the primary tumor was categorized as trunk, head/neck, upper extremities, or lower extremities. Clinical stage of all

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patients with melanoma at the time of diagnosis was reevaluated based on hospital clinical records and primary tumor histopathologic features and determined according to the 2009 AJCC Melanoma Staging and Classification.[3]

Immunohistochemistry

The immunohistochemical analysis of MMR protein expression was performed on deparaffinized tissue sections by using the monoclonal antibodies and the indirect peroxidase method previously described,[18] except that incubation with the anti-MSH6 antibody was performed at 4°C for 24 hours instead of at room temperature for 2 hours. Normal and neoplastic cells with nuclear immunohistochemical expression of MMR proteins were considered positive cells. Keratinocyte positivity[18-20] was used as adequate internal positive control for each case to validate technical procedures. For each biopsy specimen, the total number of negative or positive nevus cells or melanoma cells was counted in at least five different high-power fields. The results were pooled and the percentage of positive nevus cells or melanoma cells was calculated. Moreover, slides were evaluated independently by two blinded observers (F.P. and A.M.), and the final average of the percentages of positive cells given by the two pathologists was calculated. A good agreement was obtained between the two individual investigators with respect to the percentage of cells scored as positive. In a few cases, the percentages varied. These slides were reexamined by both pathologists and a consensus was obtained. Melanoma cases were divided into 10 classes based on the percentage of stained tumor cells (first class, 0%-10% positive cells; 10% increment for the subsequent classes).

Patients' Vital Status

Files from the Registry Office of the Lazio Region were examined to obtain information on vital status and cause-specific death of patients. The *International Classification of Diseases, Ninth Revision,* was used to classify death due to cancer (codes 172.0-172.9). The length of follow-up for each patient was calculated as the number of days from the diagnosis of primary melanoma to the date of death or to the end of the follow-up. Patients who were alive or had died of other causes were considered censored.

Statistical Analysis

Groups were compared with the Pearson χ^2 test or Fisher exact test for categorical variables and with the Kruskal-Wallis test for continuous variables. The Kaplan-Meier method was used to calculate melanoma survival (MS) by patient baseline characteristics and by levels of tumor thickness, histologic type, site of tumor, presence of ulceration, regression, mitotic rate, and MMR protein expression, with statistical *P* values generated by the Cox-Mantel log rank test. We assessed the potential for violation of the proportional hazards assumption by comparing the survival curves for each level of a variable. The multivariate Cox proportional hazards model was used to test for the independent relationship between MSH6 protein expression and melanoma mortality. Demographic and clinicopathologic factors were considered possible confounding factors. For analysis purposes, MMR protein expression levels were dichotomized based on percentiles of the distribution. Statistical significance was set at $P < .05$. Data were analyzed with STATA software (Stata 11.0; StataCorp LP, College Station, TX).

Table 1
Patients' Demographic and Clinicopathologic Data

Characteristic	No. (%)
Patients with melanoma (n = 101)	
Sex	
Male	58 (57.4)
Female	43 (42.6)
Age, y	
≤49	39 (38.6)
50-64	33 (32.7)
≥65	29 (28.7)
Breslow thickness, mm	
≤1.00	33 (32.6)
1.01-2.00	24 (23.8)
2.01-4.00	20 (19.8)
>4.00	24 (23.8)
Anatomical site	
Head/neck	13 (12.9)
Trunk	37 (36.6)
Upper limb	22 (21.8)
Lower limb	29 (28.7)
Histologic type	
Superficial spreading	62 (61.4)
Nodular	39 (28.6)
Ulceration	
Absent	87 (86.1)
Present	14 (13.9)
Regression	
Absent	93 (92.1)
Present	8 (7.9)
Mitoses/mm^2	
<1	42 (41.6)
≥1	58 (57.4)
NE	1 (1.0)
Growth phase	
Radial	9 (8.9)
Vertical	92 (91.1)
AJCC clinical stage	
IA, IB	57 (56.4)
IIA, IIB, IIC	39 (38.6)
III	4 (4.0)
NA	1 (1.0)
Patients diagnosed with CMN (n = 18)	
Sex	
Male	9 (50.0)
Female	9 (50.0)
Age, y	
≤49	7 (38.9)
50-64	6 (33.3)
≥65	5 (27.8)

AJCC, American Joint Committee on Cancer; CMN, compound melanocytic nevus; NA, not available; NE, not evaluable.

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Results

Expression of MMR Proteins in CMNs and Primary Melanomas

Immunohistochemical analysis of MSH2, MSH6, MLH1, and PMS2 protein expression was carried out on 18 specimens of CMN and on the primary tumors of 101 patients with stage I to III melanoma.

In all CMNs, nuclear immunostaining for MSH2, MLH1, and PMS2 was detected in more than 90% of the melanocytic population **Image 1** (and data not shown). On the other hand, immunoreactivity for MSH6 was either absent or detectable in a few nevus cells at the dermal-epidermal junction (Image 1 and data not shown). In agreement with previous findings,[18-20] positive staining for the four proteins was detected in normal epidermis present in the sections of CMNs. Nuclear immunoreactivity for all the MMR proteins was found in epidermal keratinocytes predominantly in the basal and first one to three suprabasal cell layers. Single scattered cells positive for MSH2, MLH1, and PMS2 were also detected in the uppermost epidermal layers (Image 1). Dermal fibroblasts, as well as endothelial cells of dermal vessels and pericytes or smooth muscle cells in the microvascular wall, showed either no or only weak immunoreactivity for the MMR proteins (data not shown).

Expression of the four MMR proteins in primary melanomas was initially categorized into 10 classes based on the percentage of positive tumor cells. The number of tumor samples within each class is illustrated in **Table 2**. Among the 101 primary melanomas, 68 (67.3%) and 77 (76.2%) samples showed positive immunostaining for MLH1 and PMS2, respectively, in more than 80% of the tumor cells. Most of the remaining cases displayed MLH1 and PMS2 immunostaining in 71% to 80% of the tumor cells, whereas only a limited number of biopsy specimens showed immunoreactivity in 70% or less of the melanoma cells. MSH2 expression showed a more heterogeneous pattern. Indeed, 38 (38.4%) tumor samples showed a percentage of positive tumor cells higher than 80%, 29 (29.3%) specimens displayed immunostaining in 71% to 80% of the tumor cells, and 32 (32.3%) melanomas showed a percentage of positive tumor cells of 70% or less. None of the cases was negative for MSH2. A different pattern of distribution was observed for MSH6, with 62.8% of the melanomas showing positive immunostaining in 20% or less of the tumor cells and most of the remaining cases displaying a percentage of positive tumor cells between 21% and 60%. In each MSH6-positive melanoma, the percentage of cells staining positive for MSH6 was lower than that of cells staining positive for MLH1 and PMS2, as well as lower than that of MSH2-positive cells, with the exception of three cases in which MSH6 and MSH2 were equally expressed. Among the samples included in the lowest expression class (ie, 0%-10%), six were negative. Representative images of MMR protein expression in primary melanomas are illustrated in Image 1.

Association Between MMR Protein Expression and Demographic and Clinicopathologic Variables

The χ^2 or Fisher exact test was used to study the association between the expression of each MMR protein and clinicopathologic variables. MMR protein expression levels were dichotomized based on percentiles of the distribution. Briefly, MSH2, MLH1, and PMS2 expression in primary melanomas was recorded as high when the percentage of positive tumor cells was more than 80% and as low when it was 80% or less. MSH6 expression was considered high when the percentage of positive tumor cells was more than 20% and low when it was 20% or less.

A statistically significant association was observed between MSH6 expression and Breslow thickness, ulceration, mitotic rate, and clinical stage **Table 3**. High MSH6 expression was found mainly in melanomas with a Breslow thickness of more than 2 mm (63.9%) compared with melanomas with a Breslow thickness of 2 mm or less (36.1%) (P = .002). Ulceration was present in 22.2% of melanomas with high MSH6 expression (H-MSH6 melanomas) and in only 6.6% of melanomas with low MSH6 expression (L-MSH6 melanomas) (P = .05). High mitotic rate (ie, mitoses/mm^2 ≥1) was also more frequent among H-MSH6 melanomas (77.8%) in comparison with L-MSH6 melanomas (P = .003). High MSH6 expression was also associated with AJCC clinical stages II to III (P = .003). No significant associations were observed between the expression of MSH2, MLH1, PMS2, and clinicopathologic variables.

Melanoma Survival

Seventy-seven of the 101 patients with melanoma included in the study were residents in the Lazio Region and had complete information on vital status. During the observation period, 19 deaths occurred, 13 of which were due to melanoma. The median follow-up time was 9.3 years (range, 8.3 months to 10.9 years).

Table 4 shows the 10-year MS according to demographic and clinicopathologic variables. Patients with nodular melanoma had lower MS compared with patients with superficial spreading melanoma (70.2% vs 89.4%, P = .01). Significantly lower survival was also observed for patients whose tumor had a Breslow thickness of more than 4 mm or mitoses/mm^2 of 1 or more compared with patients whose tumor had a Breslow thickness of 4 mm or less or mitoses/mm^2 of less than 1 (50.5% vs 91.4%, P < .0001, and 63.3% vs 100%, P < .001, respectively). In addition, MS was significantly lower in patients with stage II to III disease than

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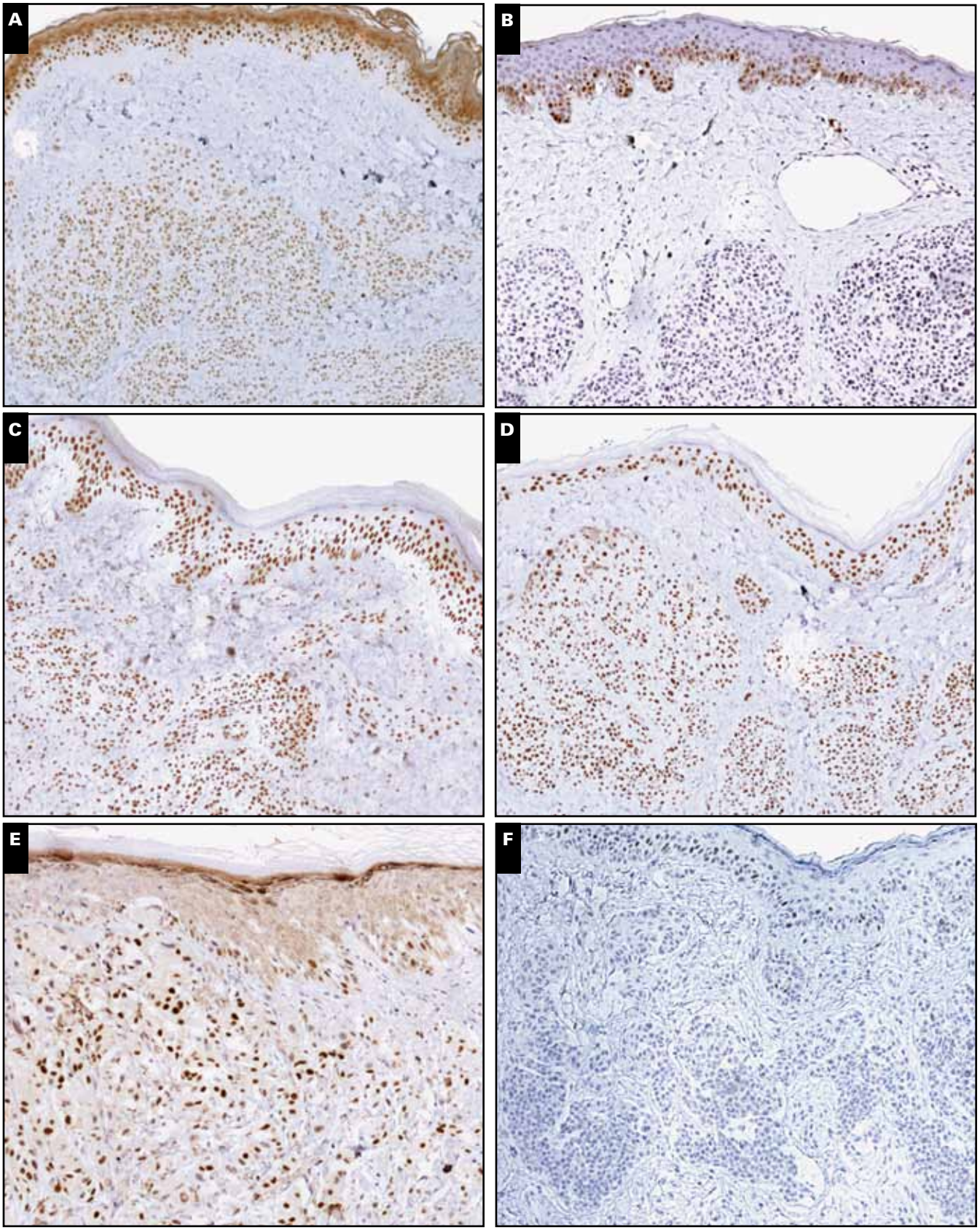

Image 1 Representative images of MSH2 (**A**), MSH6 (**B**), MLH1 (**C**), and PMS2 (**D**) expression in compound melanocytic nevi (Pt13) and of MSH6 expression in primary melanomas (Pt5 and Pt79). **E**, Pt5, positive immunostaining for MSH6 protein in 45% of tumor cells. **F**, Pt79, positive immunostaining for MSH6 protein in less than 10% of tumor cells. Immunostaining of mismatch repair proteins was performed by the indirect peroxidase method. Sections were lightly counterstained with hematoxylin (×100). Pt, patient.

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Table 2
Mismatch Repair Protein Expression in 101 Primary Melanomas

	Percentage of Positive Cells[a]									
Protein	**0-10**	**11-20**	**21-30**	**31-40**	**41-50**	**51-60**	**61-70**	**71-80**	**81-90**	**91-100**	**NE**
MSH2	3 (3.0)	4 (4.0)	1 (1.0)	7 (7.1)	4 (4.0)	5 (5.1)	8 (8.1)	29 (29.3)	22 (22.2)	16 (16.2)	2
MSH6	43 (44.3)	18 (18.5)	11 (11.3)	9 (9.3)	6 (6.2)	6 (6.2)	2 (2.1)	2 (2.1)	0	0	4
MLH1	0	0	0	0	2 (2.0)	3 (3.0)	7 (6.9)	21 (20.8)	38 (37.6)	30 (29.7)	0
PMS2	0	0	1 (1.0)	0	1 (1.0)	1 (1.0)	6 (5.9)	15 (14.8)	33 (32.7)	44 (43.6)	0

[a] Values indicate the number of samples in the indicated class of expression, with the number in parentheses representing the percentage of the total samples analyzed subtracted from the samples that were not evaluable (NE).

Table 3
Association Between Demographic and Clinicopathologic Characteristics and Mismatch Repair Protein Expression in Primary Melanomas[a]

	MSH2 (n = 99)			MSH6 (n = 97)			MLH1 (n = 101)			PMS2 (n = 101)		
Characteristic	**Low,[b] No. (%)**	**High,[b] No. (%)**	***P* Value[c]**	**Low,[d] No. (%)**	**High,[d] No. (%)**	***P* Value[c]**	**Low,[b] No. (%)**	**High,[b] No. (%)**	***P* Value[c]**	**Low,[b] No. (%)**	**High,[b] No. (%)**	***P* Value[c]**
Sex												
Male	37 (60.7)	21 (55.3)		32 (52.5)	24 (66.7)		19 (57.6)	39 (57.4)		16 (66.7)	42 (54.6)	
Female	24 (39.3)	17 (44.7)	.60	29 (47.5)	12 (33.3)	.17	14 (42.4)	29 (42.6)	.98	8 (33.3)	35 (45.4)	.29
Age, y												
≤49	27 (44.2)	12 (31.6)		26 (42.6)	11 (30.6)		17 (51.5)	22 (32.4)		12 (50.0)	27 (35.0)	
50-64	22 (36.1)	10 (26.3)		19 (31.2)	13 (36.1)		6 (18.2)	27 (39.7)		9 (37.5)	24 (31.2)	
≥65	12 (19.7)	16 (42.1)	.06	16 (26.2)	12 (33.3)	.49	10 (30.3)	19 (27.9)	.07	3 (12.5)	26 (33.8)	.13
Breslow thickness, mm												
≤1.00	21 (34.4)	10 (26.3)		24 (39.4)	7 (19.4)		14 (42.4)	19 (27.9)		10 (41.7)	23 (29.9)	
1.01-2.00	14 (23.0)	10 (26.3)		18 (29.5)	6 (16.7)		7 (21.2)	17 (25.0)		5 (20.8)	19 (24.7)	
2.01-4.00	12 (19.6)	8 (21.1)		8 (13.1)	12 (33.3)		5 (15.2)	15 (22.1)		3 (12.5)	17 (22.0)	
>4.00	14 (23.0)	10 (26.3)	.88	11 (18.0)	11 (30.6)	.02	7 (21.2)	17 (25.0)	.56	6 (25.0)	18 (23.4)	.64
Anatomical site												
Head/neck	7 (11.5)	6 (15.8)		9 (14.8)	4 (11.0)		7 (21.2)	6 (8.8)		5 (20.8)	8 (10.4)	
Trunk	27 (44.3)	10 (26.2)		24 (39.3)	11 (30.6)		13 (39.4)	24 (35.3)		10 (41.7)	27 (35.0)	
Upper limb	11 (18.0)	11 (29.0)		11 (18.0)	11 (30.6)		7 (21.2)	15 (22.1)		2 (8.3)	20 (26.0)	
Lower limb	16 (26.2)	11 (29.0)	.29	17 (27.9)	10 (27.8)	.53	6 (18.2)	23 (33.8)	.20	7 (29.2)	22 (28.6)	.20
Histologic type												
Superficial spreading	40 (65.6)	20 (52.6)		41 (67.2)	18 (50.0)		24 (72.7)	38 (55.9)		17 (70.8)	45 (58.4)	
Nodular	21 (34.4)	18 (47.4)	.20	20 (32.8)	18 (50.0)	.09	9 (27.3)	30 (44.1)	.10	7 (29.2)	32 (41.6)	.34
Ulceration												
Absent	54 (88.5)	31 (81.6)		57 (93.4)	28 (77.8)		29 (87.9)	58 (85.3)		19 (79.2)	68 (88.3)	
Present	7 (11.5)	7 (18.4)	.34	4 (6.6)	8 (22.2)	.05	4 (12.1)	10 (14.7)	>.99	5 (20.8)	9 (11.7)	.31
Regression												
Absent	55 (90.2)	36 (94.7)		58 (95.1)	32 (88.9)		31 (93.9)	62 (91.2)		21 (87.5)	72 (93.5)	
Present	6 (9.8)	2 (5.3)	.71	3 (4.9)	4 (11.1)	.42	2 (6.1)	6 (8.8)	>.99	3 (12.5)	5 (6.5)	.39
Mitoses/mm^2												
<1	27 (45.0)	13 (34.2)		32 (52.5)	8 (22.2)		16 (48.5)	26 (38.8)		10 (43.5)	32 (41.6)	
≥1	33 (55.0)	25 (65.8)	.29	29 (47.5)	28 (77.8)	.003	17 (51.5)	41 (61.2)	.36	13 (56.5)	45 (58.4)	.87
Growth phase												
Radial	6 (9.8)	1 (2.6)		7 (11.5)	1 (2.8)		3 (9.1)	6 (8.8)		4 (16.7)	5 (6.5)	
Vertical	55 (90.2)	37 (97.4)	.25	54 (88.5)	35 (97.2)	.25	30 (90.9)	62 (91.2)	>.99	20 (83.3)	72 (93.5)	.21
AJCC clinical stage												
I	35 (58.3)	20 (52.6)		42 (68.9)	13 (37.1)		21 (63.6)	36 (53.7)		15 (62.5)	42 (55.3)	
II-III	25 (41.7)	18 (47.4)	.58	19 (31.1)	22 (62.9)	.003	12 (36.4)	31 (46.3)	.35	9 (37.5)	34 (44.7)	.53

AJCC, American Joint Committee on Cancer.
[a] Totals may differ because of missing values.
[b] Low: positive tumor cells 80% or less; high: positive tumor cells more than 80%.
[c] Pearson χ^2 test or Fisher exact test, where appropriate.
[d] Low: positive tumor cells 20% or less; high: positive tumor cells more than 20%.

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in patients with stage I disease (survival 59.5% vs 95.7%, $P < .001$). No statistically significant difference in survival was found for patient sex and age, tumor location, ulceration, regression, and growth phase.

Table 5 shows MMR protein expression, 10-year MS, and crude relative risk (RR). MS was significantly lower in patients with H-MSH6 melanomas compared with patients with L-MSH6 tumors (67.9% vs 88.5%, $P = .007$). Patients whose primary melanoma was classified as H-MSH6 had a fourfold increased RR of mortality (RR, 4.39; 95% confidence interval [CI], 1.35-14.30). Kaplan-Meier survival curves for patients with H-MSH6 or L-MSH6 melanoma are

Table 4
Demographic and Clinicopathologic Data and 10-Year Melanoma Survival

Characteristic	Patients (n = 77), No. (%)	Survivors, No.	Survival	
			%	*P* Value[a]
Sex				
Male	44 (57.1)	37	83.9	
Female	33 (42.9)	27	79.7	.77
Age, y				
≤49	26 (33.8)	23	86.5	
50-64	26 (33.8)	19	73.1	
≥65	25 (32.4)	22	87.1	.29
Breslow thickness, mm				
0.3-4	57 (74.0)	53	91.4	
>4	20 (26.0)	11	50.5	<.0001
Anatomical site				
Head/neck	10 (13.0)	9	90.0	
Trunk	29 (37.7)	26	89.4	
Upper limb	16 (20.8)	13	81.3	
Lower limb	22 (28.6)	16	68.2	.35
Histologic type				
Superficial spreading	45 (58.4)	41	89.4	
Nodular	32 (41.6)	23	70.2	.01
Ulceration				
Absent	68 (88.3)	58	83.5	
Present	9 (11.7)	6	66.7	.11
Regression				
Absent	71 (92.2)	58	80.0	
Present	6 (7.8)	6	100.0	.27
Mitoses/mm^2				
<1	37 (48.0)	37	100.0	
≥1	40 (52.0)	27	63.3	<.001
Growth phase				
Radial	8 (10.4)	8	100.0	
Vertical	69 (89.6)	56	79.3	.19
AJCC clinical stage				
I	46 (59.7)	44	95.7	
II-III	31 (40.3)	20	59.5	<.001

AJCC, American Joint Committee on Cancer.
[a] Log rank test.

Table 5
Mismatch Repair Protein Expression, 10-Year Melanoma Survival, and Crude Relative Risk of Melanoma Mortality

Protein Expression	Patients (n = 77),[a] No. (%)	Survivors, No.	Survival		RR (95% CI)[c]	*P* Value
			%	*P* Value[b]		
MSH2						
Low (0-80)	47 (62.7)	40	83.2		1 [Reference]	
High (81-100)	28 (37.3)	22	78.4	.44	1.53 (0.51-4.56)	.45
MSH6						
Low (0-20)	47 (62.7)	43	88.5		1 [Reference]	
High (21-100)	28 (37.3)	19	67.9	.007	4.39 (1.35-14.30)	.01
MLH1						
Low (0-80)	22 (28.6)	19	86.4		1 [Reference]	
High (81-100)	55 (71.4)	5	79.4	.58	1.43 (0.39-5.20)	.59
PMS2						
Low (0-80)	16 (20.8)	13	81.3		1 [Reference]	
High (81-100)	61 (79.2)	51	81.3	.83	0.87 (0.24-3.16)	.83

[a] Totals may differ from 77 because of missing values.
[b] Log rank test.
[c] Crude relative risk (RR) and 95% confidence interval (CI), evaluated by the Cox proportional hazards model.

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shown in **Figure 1**. No difference in survival was found for expression levels of MSH2, MLH1, and PMS2 (Table 5).

Using the Cox proportional hazards model, multivariate analyses were performed to assess the independent predictive value of MSH6 protein expression among the primary melanomas. **Table 6** shows the estimated RR of melanoma death and 95% CIs. The presence of ulceration and high mitotic rate were strongly associated (P = .002), as well as Breslow thickness and histologic type (P < .0001). To avoid multicollinearity, ulceration and mitotic rate were not included simultaneously in the multivariate models. After controlling for age and sex, Breslow thickness, and ulceration, MSH6 expression remained an independent prognostic factor for melanoma mortality (RR, 3.76; 95% CI, 1.12-12.70; P = .03). Other variables such as anatomical site, regression, and growth phase were also considered one at a time in the models, but the results did not change.

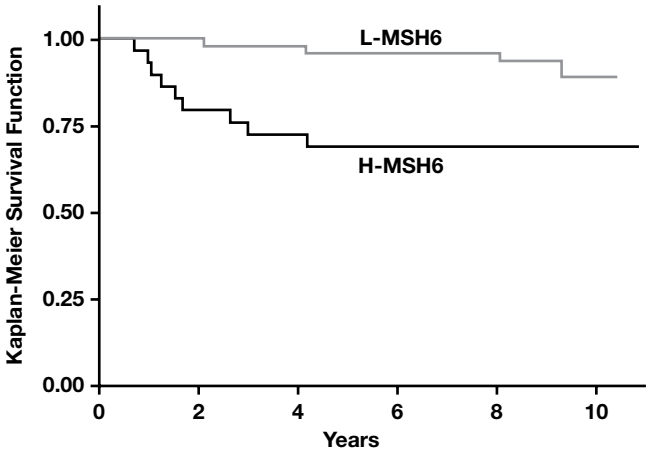

Figure 1 Melanoma survival in patients with stage I to III disease according to MSH6 protein expression in the primary tumor. H-MSH6, positive tumor cells more than 20%; L-MSH6, positive tumor cells 20% or less. P = .007.

Discussion

Germline mutations affecting DNA repair genes have been clearly associated with a high risk of cancer development.[21] On the other hand, gene expression profiling of tumor cell lines and specimens of different histologic origin has evidenced that overexpression of DNA repair genes is frequently associated with more aggressive behavior of cancer cells and/or with lower patient disease-free or overall survival.[22] These findings have led to the hypothesis that while genetic instability is essential for tumor initiation, it may be deleterious in the progression or metastatic process.[22]

The MMR system is a DNA repair pathway dedicated to the correction of replication errors that escape processing by the proofreading activity of the replicative DNA polymerase.[15,16] In eukaryotic cells, the key proteins involved in MMR are MSH2, MSH6, MLH1, and PMS2.[15,16] The protein complex MutSα, a heterodimer of MSH2 and MSH6, initially recognizes and binds base/base mismatches and insertion/deletion loops of one to three nucleotides. After this step, the heterodimer of MLH1 and PMS2, termed MutLα, interacts with the DNA-bound MutSα to initiate the repair process, which also requires additional proteins involved in DNA metabolism (eg, helicases, exonucleases).[15,16] MMR proteins are also involved in DNA damage signaling, in the processing of modified bases, and can interact with partners involved in other pathways of DNA repair.[15,16] Mutations in MMR genes confer genetic instability and are implicated in hereditary nonpolyposis colon cancer.[15,16] Notably, Winnepenninckx et al[8] and Kauffmann et al[10] found that both *MSH2* and *MSH6* were among the genes overexpressed in primary melanomas that had metastasized within 4 years from diagnosis compared with those that had not. Elevated levels of *MSH2* and *MSH6* mRNA in primary melanomas have also been described by Jewell et al[12] as being associated with unfavorable histopathologic features. Finally, overexpression of *MSH6* occurred in primary melanomas classified by Harbst et al[13] as "high-grade" melanomas, according to a two-class signature constructed through gene expression profiling of a large set of tumor specimens. Compared with "low-grade"

Table 6
MSH6 Expression and Melanoma Mortality: Adjusted Relative Risk (RR) and 95% Confidence Intervals (CIs) for High MSH6 Expression (H-MSH6)

	Melanoma Mortality	
Multivariate Model	**RR (95% CI)[a]**	***P* Value**
Model a: H-MSH6, sex, age	4.60 (1.40-15.10)	.01
Model b: model a plus Breslow thickness	3.82 (1.15-12.70)	.03
Model c: model a plus presence of ulceration	4.23 (1.27-14.10)	.02
Model d: model a plus Breslow thickness and ulceration	3.76 (1.12-12.70)	.03

[a] Evaluated by the Cox proportional hazards model.

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Table 7
Studies That Have Analyzed the Expression of One or More MMR Proteins in Both Benign Nevi and Primary Melanomas

Author	Samples[a]	Protein[b]	Protein Expression in BNs	Protein Expression in PMs
Korabiowska et al[23]	51 BNs and 78 PMs	MSH2, MLH1, and PMS2	The three proteins were expressed in most samples, even though there were appreciable differences in the percentage of positive cells.	Only 37% of the samples displayed positive immunostaining for all the proteins.
Hussein et al[19]	19 BNs and 9 PMs	MSH2, MSH6, and MLH1	The three proteins were highly expressed in all samples.	The percentage of cells staining positive for any of the proteins was reduced compared with BNs.
Rass et al[24]	8 BNs and 15 PMs	MSH2	The protein was absent (one case) or present in a few scattered nevus cells.	All samples displayed positive immunostaining for the protein in almost the entire tumor cell population.
Alonso et al[25]	10 BNs and 94 PMs	MSH2 and MLH1	Almost all samples showed MSH2- and MLH1-positive staining in more than 70% and 50% of the nevus cells, respectively.	A few samples showed reduced expression of the two proteins compared with BNs.
Song et al[26]	53 BNs and 312 PMs	MSH2	The protein was expressed in all samples with different histoscore values.	The protein was expressed in all samples with different histoscore values. The median value of the MSH2 histoscore did not differ significantly from that of BNs.

[a] Number of benign nevi (BNs) and primary melanomas (PMs) examined.
[b] The expression of the indicated mismatch repair (MMR) proteins was evaluated by immunohistochemistry.

melanomas, "high-grade" tumors were associated with negative clinicopathologic prognostic factors and poorer relapse-free survival and overall survival.[13] On these bases, in the present study we analyzed the expression of MSH2, MSH6, MLH1, and PMS2 proteins in CMNs and in a large series of primary melanomas, as well as investigated its role as a prognostic factor in patients with stage I to III disease.

To our knowledge, only five studies,[19,23-26] briefly described in **Table 7**, have analyzed the expression of one or more MMR proteins in both benign nevi and primary melanomas, with some contrasting results. Overall, the results of our study, which is the first to evaluate the expression of all four key MMR proteins in the same set of benign nevi and primary melanomas, support previous findings showing that MSH2, MLH1, and PMS2 proteins are expressed in most nevus cells and that, with respect to benign nevi, a subset of primary melanomas displays reduced immunostaining for one or more of these MMR proteins. Differently from Hussein et al,[19] we detected an extremely low MSH6 immunoreactivity in benign nevi and increased MSH6 expression in a subset of primary melanomas. Notably, none of the CMNs displayed MSH6 immunoreactivity in the dermal portion of the lesion, whereas dermal expression of MSH6 was always present in MSH6-positive primary melanomas. The expression pattern of MSH6 in benign nevi and primary melanomas partially resembles that reported by previous studies for several proliferation markers, including Ki-67, topoisomerase II-α, and proliferating cell nuclear antigen. In benign nevi, the percentage of melanocytes expressing these markers has been shown to be very low, ranging between 0% and 4%,[27-32] whereas in primary melanomas, the percentage of tumor cells staining positive for proliferation markers has been reported to range between 5% and 50%.[27,28,31,33] Moreover, in CMNs, proliferating nevus cells were located in the junctional component[28,29,31] or in the mid-dermal portion[27,30] of the nevus, whereas in primary melanomas, they were evidenced also in the deep dermal tumor nests.[28,32,33] We can hypothesize that in both benign nevi and primary melanomas, MSH6 is expressed only in proliferating cells, whereas MSH2, MLH1, and PMS2 are also present in resting cells. This hypothesis is further supported by the finding that the expression of MSH6 but not that of MSH2, MLH1, or PMS2 was significantly associated with the mitotic rate of primary melanomas.

With respect to the relationship between MMR protein expression and clinicopathologic variables, our data show that high MSH6 expression in primary melanomas was significantly associated with unfavorable histopathologic features—namely, tumor thickness of more than 2 mm, ulceration, and number of mitoses/mm^2 of 1 or more—and with clinical stages II to III. Patients with H-MSH6 melanomas showed a lower rate of survival (67.9%) compared with patients with L-MSH6 melanomas (88.5%). In the univariate analysis, patients with H-MSH6 melanomas had a risk of death fourfold higher than that of patients with L-MSH6 tumors. More important, MSH6 expression was independent of age and sex, Breslow thickness, and ulceration in the prediction of 10-year melanoma mortality in the multivariate Cox regression analysis. In the multivariable analysis, Breslow thickness remained statistically significant. These results confirm, at the protein level, previous data from gene expression profiling showing high levels of *MSH6*

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mRNA in more aggressive primary melanomas.[8,10,12,13] In our study, neither MSH2 expression nor that of MLH1 and PMS2 was significantly associated with any clinicopathologic parameter or patient 10-year MS. In a recent investigation by Meyer et al,[34] MSH2 and MLH1, but not MSH6 and PMS2, were included in a panel of 70 proteins whose expression was evaluated by immunohistochemistry in the primary melanoma of 364 patients to identify a multimarker signature predictive of clinical outcome. In this investigation, high MLH1 but not high MSH2 expression was associated with a high risk of melanoma mortality in the univariate analysis. Song et al[26] reported that patients with primary melanomas displaying high MSH2 protein levels did not show lower survival compared with patients whose tumors had low MSH2 expression. Regarding MSH2 expression, our data are consistent with these previous findings and further confirm that even though overexpression of *MSH2* mRNA has been detected in primary melanomas with unfavorable histopathologic features and/or poor clinical outcome,[8,10,12] the expression of MSH2 protein is not a prognostic factor for melanoma mortality. On the other hand, it cannot be excluded that we did not evidence an association between MLH1 expression and melanoma mortality as a result of the lower number of patients included in our study with respect to that considered by Meyer and colleagues.[34]

As discussed above, the staining pattern of MSH6 in benign nevi and primary melanomas supports the hypothesis that this protein is expressed in proliferating but not resting nevus or tumor cells. Elevated mitotic rate (histologically defined as mitoses/mm^2 $\geq$1) has been demonstrated to be an important independent adverse predictor of survival in melanoma, and in the seventh edition of the AJCC Melanoma Staging and Classification, it has replaced the Clark level of invasion as the primary criterion for defining the T1b subcategory.[3] High expression of the Ki-67 proliferation markers in primary melanoma also has been clearly associated with poor clinical outcome (reviewed in Gould Rothberg et al[35] and Moore et al[36]). The prognostic value of high MSH6 expression in primary melanomas can therefore be in part dependent on its association with proliferation. However, for metastatic spreading, which ultimately determines patient outcome, tumor cells must be able not only to proliferate but also to leave the original site, enter the bloodstream, reach a distant organ, extravasate, and grow. Accordingly, in several types of malignancies, a large number of genes involved in different biological processes beside proliferation appear to be differentially expressed between tumors that had developed metastasis and tumors that had not.[6,7] Many DNA repair genes are among those genes frequently overexpressed in cancer that are going to metastasize, and it has been suggested that this ensures highly proliferating cells protection from DNA damage, promoting invasiveness and survival in unfavorable environments.[22] The MSH6 protein not only is required for a functional MMR system, but emerging evidence also supports a key role for this protein in MMR-dependent DNA damage response and in the interaction between MMR and other DNA repair pathways within the cells.[37] For instance, MSH6 has been reported to interact with Ku70 and to promote DNA double-strand break repair by nonhomologous end joining.[37] It also associates with the Bloom syndrome helicase, which plays an important role in the repair of DNA double-strand breaks by homologous recombination.[37] MSH6 expression in proliferating cells could therefore confer protection against different types of DNA damage, promoting survival and, hence, a successful metastatic spreading. Future studies aimed at a deep molecular characterization (eg, whole-exome sequencing, gene expression profiling) of primary melanomas with "high" or "low" expression of MSH6 protein might disclose the key molecular pathways underlying the higher aggressiveness of the tumors with the H-MSH6 phenotype. It is worth noting that although tumor cells expressing a functional MMR are more protected against a variety of DNA damages, they can be susceptible to the cytotoxic effects of chemotherapeutic methylating agents able to form adducts at the O^6 position of guanine (ie, dacarbazine, temozolomide). O^6-methylguanine (O^6-MeG) in DNA can be repaired by the enzyme O^6-methylguanine-DNA methyltransferase (MGMT), which is expressed at variable levels in tumor cells.[38-40] In the absence of repair, however, O^6-MeG frequently mispairs with thymine during DNA duplication. O^6-MeG:T mismatches can be then recognized by the MMR system, which activates a signaling cascade resulting in cell cycle arrest at the G_2 phase of the second cell doubling event, which is followed by apoptosis, mitotic catastrophe, or a senescence-like state.[41-46] In MMR-proficient cells, sensitivity to O^6-guanine-methylating agent is strictly dependent on the level of MGMT, whereas cells with a defective MMR are highly resistant to these agents, regardless of their MGMT activity.

In conclusion, our study provides further insight into the expression pattern of MSH2, MSH6, MLH1, and PMS2 proteins in benign nevi and primary melanomas and suggests that the level of MSH6 protein expression can provide useful prognostic information beyond that provided by routine clinical and histologic factors. Additional studies by other investigators are warranted to definitely ascertain whether MSH6 protein expression can be used as an additional independent prognostic indicator in primary melanoma to identify patients at higher risk for death due to the disease, who would then be candidates for adjuvant therapy.

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